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TITLE OF INVENTION (500 characters max)

FUSION POLYPEPTIDES OF HUMAN FETUIN AND THERAPEUTICALLY ACTIVE POLYPEPTIDES

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ENCLOSED APPLICATION PARTS (check all that apply)

☒ Specification 96 pages, including title page (not numbered), text (59 pages, pages 2-60), claim (1 page, page 61, 1 claim), abstract (1 page, page 62), Table 1 (2 pages, pages 63-64), Tables 2 and 3 (1 page, page 65), and Appendix A (16 pages, pages 66-81), Appendix B (7 pages, pages 82-88), and Appendix C (5 pages, pages 89-93); and

☒ Drawings: 3 Sheets, Figures 1-2.

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Respectfully submitted,

SIGNATURE _____ Date February 18, 2005

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UNITED STATES PROVISIONAL PATENT APPLICATION

for

FUSION POLYPEPTIDES OF HUMAN FETUIN AND THERAPEUTICALLY ACTIVE POLYPEPTIDES

by

KETING CHU

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I. FIELD OF THE INVENTION

[001] The invention relates to novel fetuin molecules, naturally occurring splice variants of fetuin molecules, fetuin fusion proteins, polynucleotides that encode fetuin fusion proteins, as well as vectors, host cells, and compositions containing fetuin fusion proteins. The invention also relates to methods of producing fetuin fusion proteins and methods of using them to enhance the effects of therapeutic polypeptides.

II. BACKGROUND OF THE INVENTION

A. FETUINS

[002] Fetuins are low molecular-weight globulins that constitute a large portion of the globulins in fetal blood and are members of the cystatin superfamily (Brown et al, 1992). Fetuin A, also known as alpha 2HS glycoprotein (alpha 2HS), and fetuin B are found on chromosome 3q27, and share conserved cysteine residues at certain positions. Both have two tandemly arranged cystatin protein domains (http://biostatpub2.md anderson.org/cgi-bin/genecards/cardsearch.pl?search=fetuin). Fetuin A and fetuin B are transcribed as precursor proproteins from a single mRNA, then cleaved to form two polypeptides, which remain linked by a connecting sequence. Fetuin A is transcribed from a 1538 bp mRNA (NM_001622) and fetuin B is transcribed from a 1623 bp mRNA (NM_014375) (National Center for Biotechnology Information (NCBI)).

[003] Fetuins are multifunctional proteins. They play a role in calcium homeostasis; deliver fatty acids to, and remove cholesterol from, fetal cells; regulate the formation of a fertilizable egg; and play a role in acute inflammatory responses (Wang et al., 1998). They also antagonize the antiproliferative action of transforming growth factor beta (TGFβ) and induce apoptosis to exert anti-cancer activity (Yu et al., 2001). Fetuins characteristically have two

tandem cystatin domains, which inhibit cysteine peptidases of the papain family, at their amino terminal ends. (http://pfam.wustl.edu/cgi-bin/getdesc?name=Cystatin).

[004] Human fetuin A is a secreted protein weighing 39,324 daltons, and having 367 amino acids (NCBI NP_001613). It has two common alleles, AHSG*1 and AHSG*2. AHSG*1 is characterized by ACG, which encodes threonine, at position 230 and ACC, which also encodes threonine, at position 238. ASHG*2 is characterized by ATG, which encodes methionine, at position 230 and AGC, which encodes serine, at position 238 (Osawa et al., 1997). Human fetuin B is a secreted protein weighing 42,094 daltons, and having 382 amino acids (NCBI NP_055190). It is expressed specifically in the liver (Olivier et al., 1999).

[005] Fetuin has been reported to be co-expressed *in vitro* with a sequence of interest (Chan et al, 2000). For example, a fetuin expression vector can be used to transform a mammalian host cell, e.g., a CHO cell, that expresses the sequence of interest when it is transformed with the expression vector. Fetuin has also been reported to bind to cytokines of the TGFβ superfamily and to inhibit the activity of TGFβ (Dennis and Demetriou, 1998). The TGFβ binding domain of fetuin has been characterized and described to show similarity to the TGFβ receptor II homology 1 domain of the TGFβ receptor (Dennis and Demetriou, 1998). A chimeric molecule comprising fetuin with its TGFβ binding domain replaced by the TGFβ receptor II homology 1 domain has been reported to be useful in determining if a substance modulates a protein in the TGFβ superfamily (Dennis and Demetriou, 1999; Dennis and Demetriou, 1998).

B. CYTOKINES

[006] Cytokines, extracellular signaling proteins or peptides, act as local mediators in communication among cells. Cytokines regulate proliferation and differentiation; for example,

they mediate differentiation of cells in the hematopoeitic lineage. Examples of cytokines include interleukins, interferons, and colony stimulating factors of the hematopoeitic system. Some cytokines, e.g., interferons and interleukins, can be induced by viral activity, and possess antiviral activity (Sheppard et al., 2003).

[007] Cytokines are used as therapeutic proteins in the treatment of a variety of disorders, including cancer, immune disorders, and inflammation. For example, erythropoietin regulates red cell production, promotes erythroid differentiation, initiates hemoglobin synthesis, protects from and reverses diabetic neuropathy, facilitates recovery from spinal cord injury, and provides neuroprotective therapy in brain injury (Bianchi et al., 2004; Celik et al., 2002; Siren et al., 2001; Eschbach et al., 1987). It thus provides a therapeutic tool for modulating the course of these disorders.

[008] Colony-stimulating factors mediate the survival, proliferation, and differentiation of hematopoietic progenitor cells. The actions of colony stimulating factors also illustrate the therapeutic uses of cytokines. For example, granulocyte-macrophage colony stimulating factor (GM-CSF), also known as CSF2, can modulate myeloproliferative disorders (Birnbaum et al., 2000). Granulocyte colony stimulating factor (G-CSF), also known as CSF3, specifically stimulates the proliferation and differentiation of the progenitor cells for granulocytes. It has been reported to mobilize hematopoietic progenitor stem cells in clinical transplantation, in part by its action in reducing cytokine stromal cell-derived factor 1 levels in bone marrow (Petit et al., 2002). Also by way of example, IL-11 raises platelet counts in thrombocytopenic patients (Bast et al., 2000).

[009] The cytokine B-lymphocyte stimulator (BlyS), also known as tumor necrosis factor ligand superfamily member 13B, is a cytokine that has been reported to induce activation,

proliferation, differentiation, survival, and death in B cells (Claudio et al., 2002; Moore et al., 1999). It presents a therapeutic target in disorders involving B cells. For example, it can play an antiapoptotic role in B-cell tolerance loss (Zhang et al., 2001), and can promote the survival and maturation of B2-type memory B cells (Schiemann et al., 2001). Patients with systemic lupus erythematosus (SLE) have increased levels of BlyS, and it has been reported that BlyS may provide therapy for SLE and other autoimmune diseases, e.g., rheumatoid arthritis, psoriasis, psoriatic arthritis, and ankylosing spondylitis (Zhang et al., 2001).

[010] These examples demonstrate that cytokines and other secreted regulatory factors have been used successfully in the clinic to treat disease conditions. However, their efficacy and usefulness have been previously limited by their relatively short plasma stability half life and the need for frequent dosing.

C. FUSION PROTEINS ENHANCE THERAPEUTIC EFFICACY

[011] Gene manipulation techniques have enabled the development and use of recombinant therapeutic proteins with fusion partners that impart desirable pharmacokinetic properties. Recombinant human serum albumin fused with synthetic heme protein has been reported to reversibly carry oxygen (Chuang et al., 2002). The long half-life and stability of human serum albumin (HSA) make it an attractive candidate for fusion to short-lived therapeutic proteins (Fleer et al., 2004).

[012] For example, the short plasma half-life of unmodified interferon alpha makes frequent dosing necessary over an extended period of time, in order to treat viral and proliferative disorders. Interferon alpha fused with HSA has a longer half life and requires less frequent dosing than unmodified interferon alpha; the half-life was 18-fold longer and the clearance rate was approximately 140 times slower (Osborn et al., 2002). Interferon beta fused

with HSA also has favorable pharmacokinetic properties; its half life was reported to be 36-40 hours, compared to 8 hours for unmodified interferon beta (Sung et al., 2003). A HSA-interleukin-2 fusion protein has been reported to have both a longer half-life and favorable biodistribution compared to unmodified interleukin-2. This fusion protein was observed to target tissues where lymphocytes reside to a greater extent than unmodified interleukin 2, suggesting that it exerts greater efficacy (Yao et al., 2004).

[013] The Fc receptor of human immunoglobulin G subclass 1 has also been used as a fusion partner for a therapeutic molecule. It has been recombinantly linked to two soluble p75 tumor necrosis factor (TNF) receptor molecules. This fusion protein has been reported to have a longer circulating half-life than monomeric soluble receptors, and to inhibit TNFα-induced proinflammatory activity in the joints of patients with rheumatoid arthritis (Goldenberg, 1999). This fusion protein has been used clinically to treat rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, and ankylosing spondylitis (Nanda and Bathon, 2004).

III. SUMMARY OF THE INVENTION

[014] The invention provides novel fetuin A molecules. The coding nucleic acid sequences are designated by SEQ. ID. NOS. 6-9 and 23-28. The corresponding peptide sequences are designated by SEQ. ID. NOS. 34-37 and 51-56. Additional nucleic acid sequences are designated by SEQ. ID. NOS. 58-59. SEQ. ID. NOS. 23-28 and SEQ. ID. NOS. 51-56 correspond to novel, naturally occurring splice variants of fetuin A derived from fetal liver and which are homologous to human alpha 2HS glycoprotein. The invention further provides a novel fetuin B molecule, designated by SEQ. ID. NOS. 17-22, SEQ. ID. NOS. 45-50, AND SEQ. ID. NO. 61.

[015] The invention provides a fusion molecule comprising a first polypeptide that comprises a first amino acid sequence of a therapeutic molecule or prophylactic molecule and a second polypeptide that comprises a second amino acid sequence of a fetuin fusion partner. The fusion molecule has a higher stability in plasma than a therapeutic molecule or prophylactic molecule in the absence of the fusion partner. The fetuin fusion partner can comprise either a fragment or the entirety of fetuin A, a naturally occurring splice variant of fetuin A, or fetuin B. Fetuin A, naturally occurring splice variants of fetuin A, and fetuin B may be of human origin. The fetuin fusion partner can comprise any variant, whether natural or engineered. The variants can comprise mutations and/or deletions, and/or additions of one or more amino acid residues. The variant may comprise a deletion in exon two of human fetuin A. Further, the variant may comprise a deletion of amino acid residue 71 of human fetuin A. The deletion may arise by natural processes, e.g., by alternative splicing in exon two, or the deletion may be constructed *in vitro* using standard genetic engineering methods. The fetuin fusion partner can comprise a mature fetuin polypeptide. The therapeutic molecule or prophylactic molecule can be a cytokine. Cytokines suitable for use in the invention include, but are not limited to, interferons, interleukins, erythropoietin, colony stimulating factors, stem cell factors, tumor necrosis factors, and vascular endothelial growth factor.

[016] The fetuin fusion partner can be linked to the N-terminus of the therapeutic or prophylactic molecule, or alternatively, the fetuin fusion partner can be linked to the C-terminus of the therapeutic or prophylactic molecule. The fetuin fusion molecule can comprise a linker, e.g., a peptide linker. The linker can comprise an amino acid sequence that is present in a precursor fetuin molecule. The linker may also comprise an enzyme cleavage site.

[017] The fetuin fusion partner can comprise a sequence of at least six consecutive amino acid residues chosen from a naturally occurring fetuin polypeptide or a homologue thereof, including all naturally occurring alleles and variants. The fetuin fusion partner can comprise an N-terminal amino acid sequence of a mature peptide of a naturally occurring fetuin. This N-terminal amino acid sequence can comprise at least six consecutive amino acid residues chosen from amino acid residues 23-132 of human fetuin A, at least six consecutive amino acid residues chosen from amino acid residues 23-131 of human fetuin A splice variant, or at least six consecutive amino acid residues chosen from amino acid residues 37-104 of human fetuin B, which exclude the signal sequence but include the first cystatin Pfam domain. This N-terminal amino acid sequence can further comprise at least six consecutive amino acid residues chosen from amino acid residues 23-247 of human fetuin A, or amino acid residues 23-246 of human fetuin A splice variant, or amino acid residues 37-254 of human fetuin B.

[018] The fetuin fusion partner can comprise a C-terminal amino acid sequence of a mature peptide of a naturally occurring fetuin. This C-terminal amino acid sequence can comprise at least six consecutive amino acid residues chosen from among amino acid residues 248-366 of human fetuin A, amino acid residues 247-365 of human fetuin A splice variant, or amino acid residues 255-382 of human fetuin B, which lack a cystatin Pfam domain. This C-terminal amino acid sequence can also comprise at least six consecutive amino acid residues chosen from among amino acid residues 150-366 of human fetuin A, amino acid residues 149-365 of human fetuin A splice variant, or amino acid residues 157-382 of human fetuin B, which contain one cystatin Pfam domain.

[019] The fetuin fusion partner can comprise a leader sequence. This leader sequence can comprise a secretion signal sequence which can be a naturally occurring signal sequence for

a therapeutic molecule or prophylactic molecule comprising the fusion protein of the invention. Alternatively, this secretion signal sequence can be a naturally occurring signal sequence for the fetuin fusion partner.

[020] The invention also provides a polynucleotide encoding the fusion molecule of any of the peptides described above. This polynucleotide may be part of a vector that also comprises a regulatory sequence for transcribing of the polynucleotide. The invention further provides a host cell comprising any of the fusion polypeptides described above, a polynucleotide encoding the fusion molecule of any of these polypeptides, or a vector that comprises a polynucleotide encoding the fusion molecule of any of these polypeptides and a regulatory sequence for transcription of the polynucleotide. The invention yet further provides a composition comprising any of the fusion polypeptides or polynucleotides described above, and/or any of the vectors or host cells described above, and a buffer or a pharmaceutically acceptable carrier.

[021] In another aspect, the invention provides a method of making a fusion molecule by providing a polynucleotide encoding a first polypeptide that comprises a first amino acid sequence of a therapeutic molecule or prophylactic molecule and a second polypeptide that comprises a second amino acid sequence of a fetuin fusion partner and expressing the polynucleotide in an expression system to produce the fusion molecule. The expression system can be a cell free expression system, a prokaryotic expression system, or a eukaryotic expression system. The prokaryote can be a bacterial cell, and the eukaryote can be an animal cell, e.g., a CHO cell or a COS cell. The eukaryote can also be a plant cell, or a yeast cell, such as a *Saccharomyces* or *Pichia* cell.

[022] The invention also provides a method of producing a polynucleotide that encodes a first polypeptide that comprises a first amino acid sequence of a therapeutic molecule or

prophylactic molecule and a second polypeptide that comprises a second amino acid sequence of a fetuin fusion partner by providing and amplifying such a polynucleotide. The amplification can be accomplished by introducing the polynucleotide into a cell and culturing the cell under conditions that allow the polynucleotide to be amplified. The amplification can also be accomplished by allowing the polynucleotide to undergo the polymerase chain reaction.

IV. BRIEF DESCRIPTION OF THE TABLES, APPENDICES, AND FIGURES

[023] Table 1 (Sequence Identification) lists the fetuin SEQ. ID. NOS. of the invention in relation to the sequences that are provided in the Appendices. Each SEQ. ID. NO. is identified by an internal identification (FP ID) number, as shown in the first column. The nucleotide sequence ID number for the open reading frame of the nucleic acid sequence (N1) is shown in the second column. The amino acid sequence ID number for the polypeptide sequence (P1) is shown in the third column. The nucleotide sequence ID number for the entire nucleic acid sequence (N0) is shown in the fourth column, and the fifth column shows the polypeptide and nucleotide (mRNA) ID numbers of the source clone or sequence; previously known sequences are identified by their public National Center for Biotechnology Information (NCBI) protein identification number (Source ID). The sixth column denotes whether the sequence relates to a fetuin A or fetuin B gene (Gene).

[024] Table 2 (Pfam Coordinates of Fetuin Sequences) lists the internal identification numbers (FP ID) in the first column. The novel and currently known forms of fetuin (Source ID) are listed in the second column. The third and fourth columns list the coordinates of the cystatin Pfam domains corresponding to each. The "Pfam" system is an organization of protein sequence classification and analysis, based on conserved protein domains; it can be publicly accessed in a number of ways, for example, at http://pfam.wustl.edu. Protein domains are portions of proteins

that have a tertiary structure and sometimes have enzymatic or binding activities; multiple domains can be connected by flexible polypeptide regions within a protein. Pfam domains can be connected by flexible polypeptide regions within a protein. Pfam domains can comprise the N-terminus or the C-terminus of a protein, or can be situated at any point in between. The Pfam system identifies protein families based on these domains and provides an annotated, searchable database that classifies proteins into families (Bateman et al., 2002). Each of the novel and currently known forms of fetuin have at least one cystatin Pfam domain.

[025] Table 3 (Signal Peptide and Transmembrane Coordinates of Fetuin Sequences) shows a comparison between the disclosed molecules and the known variants. The first column designates whether the sequence relates to the fetuin A or fetuin B gene (Gene). The second column designates the internal reference number (FP ID). The third column lists the novel molecules and currently known forms of fetuin (Source ID). The fourth column classifies each as a secreted protein. The fifth column lists the predicted length of each polypeptide in terms of the number of amino acid residues (Predicted Protein Length). The sixth column specifies the result of an algorithm that predicts whether a sequence is secreted (Treevote). This algorithm is constructed on the basis of a number of attributes that include hydrophobicity, two-dimensional structure, prediction of signal sequence cleavage site, and other parameters. A high Tree vote score indicates that the polypeptide is more likely to be secreted; the scale ranges from 0 to 1. The Tree vote scores predict that the sequences listed in the Tables are secreted polypeptides or nucleotides related to secreted polypeptides. The seventh column lists the coordinates of the signal peptides (Signal Peptide Coordinates). The eighth column lists the coordinates of the amino acid residues of the mature polypeptide after cleavage of the signal peptide sequence (Mature Protein Coordinates). The ninth and tenth columns list alternate coordinates of the

amino acid residues of the signal peptide and mature polypeptide after cleavage of the signal peptide sequence (Alternative Signal Peptide Coordinates) (Alternate Mature Protein Coordinates). In instances where the mature protein start residue overlaps the signal peptide end residue, some of the amino acid residues may be cleaved off such that the mature protein does not start at the next amino acid residue from the signal peptides, resulting in the alternative mature protein coordinates. The coordinates shown in Table 3 are listed in terms of the amino acid residues beginning with "1" at the N-terminus of the polypeptide.

[026] Appendix A provides polynucleotide sequences of the open reading frames that encode the polypeptides of the present invention "SEQ. ID. NO. (N1)." Appendix B provides the amino acid sequences of the polypeptides described herein "SEQ. ID. NO. (P1)." Appendix C provides the complete polynucleotide sequences of the invention, including both coding and non-coding regions "SEQ. ID. NO. (N0)."

[027] Figure 1 shows the amino acid sequence alignment of novel fetuins of the invention, including the naturally occurring splice variant, as compared to fetuin A, obtained from the NCBI public database. The asterisks (*) indicate shared amino acid residues. The colons (:) indicate conserved amino acid changes. The periods (.) indicate semi-conservative amino acid changes.

[028] Figure 2 shows the amino acid sequence alignment of a novel fetuin of the invention as compared to fetuin B, obtained from the NCBI public database. The asterisks (*) indicate shared amino acid residues. The colons (:) indicate conserved amino acid changes. The periods (.) indicate semi-conservative amino acid changes.

V. DETAILED DESCRIPTION OF THE INVENTION

A. DEFINITIONS

[029] The terms "polypeptide," "peptide," and "protein," used interchangeably herein, refer to a polymeric form of amino acids of any length, which can include naturally-occurring amino acids, coded and non-coded amino acids, chemically or biochemically modified, derivatized, or designer amino acids, amino acid analogs, peptidomimetics, depsipeptides, and polypeptides having modified, cyclic, bicyclic, depsicyclic, or depsibicyclic peptide backbones. The term includes single chain proteins as well as multimers. The term also includes conjugated proteins, fusion proteins, including, but not limited to, GST fusion proteins, fusion proteins with a heterologous amino acid sequence, fusion proteins with heterologous and homologous leader sequences, fusion proteins with or without N-terminal methionine residues, pegylated proteins, and immunologically tagged proteins.

[030] The terms "polynucleotide," "nucleic acid," and "nucleic acid molecule" are used interchangeably herein to refer to polymeric forms of nucleotides of any length. The polynucleotides can contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides can have any three-dimensional structure, and can perform any function, known or unknown.

[031] A "fusion partner" is a polypeptide fused in-frame at the N-terminus and/or C-terminus of a therapeutic or prophylactic polypeptide, or internally to a therapeutic or prophylactic polypeptide. For example, the fusion partner may be any fetuin, variant of fetuin, or any fragment thereof.

[032] A "leader sequence," or a "signal peptide," or a "signal sequence" comprises a sequence of amino acid residues, typically, at the N terminus of a polypeptide, which directs the

intracellular trafficking of polypeptides that are destined to be either secreted or membrane components. Leader sequences are generally hydrophobic and have some positively charged residues. Polypeptides that contain a signal peptide or leader sequence typically also contain a signal peptide or leader sequence cleavage site, which can be acted upon by a signal peptidase. Leader sequences can be natural or synthetic, heterologous, or homologous with the protein to which they are attached.

[033] A "secretion signal sequence" is a leader sequence that directs a protein to be secreted from the cell. A secretion signal sequence can be naturally occurring or it can be engineered.

[034] A "mature polypeptide" is a polypeptide that has been acted upon by a signal peptidase, for example, after being directed to an appropriate intracellular compartment such as the endoplasmic reticulum.

[035] A "peptide linker" is an amino acid sequence that is present in a precursor fetuin molecule. It may also comprise an amino acid sequence encoded by a synthetic DNA linker sequence. It may be a short amino acid sequence used to attach a first polypeptide to a second polypeptide. Also included in the definition are short amino acid sequences used to attach functional groups, e.g., polypeptides, polyamino acids, antibodies, antibody fragments, epitopes, purification tags, or branch point amino acids, to a polypeptide of the invention.

[036] A "precursor" molecule is one which precedes another or from which another is derived, such as a molecule which exists as an ingredient, reactant, or intermediate in an anabolic pathway that synthesizes a particular product. For example, profetuin is the precursor molecule for fetuin. A precursor may give rise to a more active or more mature molecule.

[037] A "variant" is a polypeptide of the invention and also comprises variations of naturally occurring proteins, where such variations are homologous or substantially similar to the naturally occurring protein, as well as corresponding homologs from different species. Variants of polypeptide sequences include insertions, additions, deletions, or substitutions, compared with the subject polypeptides. The term also includes peptide aptamers.

[038] A "variant" is also a nucleic acid of the invention. It may encode a variant polypeptide of the invention. The term also provides for alternatively spliced RNA and nucleic acids derived from alternatively spliced RNA, as well as variations of naturally occurring nucleic acids, where such variations are homologous or substantially similar to the naturally occurring nucleic acid, as well as corresponding homologs from different species. Variants of nucleic acid sequences include insertions, additions, deletions, or substitutions, compared with the subject nucleic acid. The term also includes nucleic acid aptamers.

[039] A "fragment" is derived from a polypeptide of the invention or a nucleic acid of the invention. It may correspond to a biologically active, functional or consensus domain, e.g., a cystatin domain, signal peptide, enzyme active site, DNA binding domain, regulatory domain, and the like.

[040] A "mutation" is a change in the amino acid sequence of a polypeptide when compared to a subject polypeptide. It is also a change in the nucleotide sequence of a nucleic acid when compared to a subject nucleic acid. The nucleotide sequence containing a mutation may encode a polypeptide containing a mutation. The mutation may be an insertion, deletion, substitution or rearrangement. The mutation may arise through natural processes, e.g., evolution and natural selection, or may arise through the use of standard genetic engineering methods that are well known in the art.

[041] A "deletion" is the removal of one or more amino acids from a polypetide when compared to a subject polypeptide. It is also the removal of one or more nucleotides from a nucleic acid sequence when compared to a subject nucleic acid sequence. The nucleic acid sequence containing a deletion may encode a polypeptide containing a deletion. The deletion may arise through natural processes, e.g., evolution and natural selection, or through the process of alternative splicing, or it may arise through the use of standard genetic engineering methods that are well known in the art.

[042] A "naturally occurring" sequence is one that exists in nature and without artificial aid. It can exist in any species, and includes all allelic and splice variants.

[043] A "fusion molecule" is a molecule, e.g., a polynucleotide or polypeptide, that represents the joining of all or portions of more than one gene or its products. For example, a fusion protein can be the product from splicing strands of recombinant DNA and expressing the hybrid gene. A fusion molecule can be made by genetic engineering, e.g., by removing the stop codon from the DNA sequence of the first protein, then appending the DNA sequence of the second protein in frame. That DNA sequence will then be expressed by a cell as a single protein. Typically this is accomplished by cloning a cDNA into an expression vector in frame with an existing gene.

[044] A "linker" is a fragment of synthetic DNA containing a restriction endonuclease recognition site that may be used for splicing genes. The term includes polylinkers, which contain several restriction enzyme recognition sites. A linker may be part of a cloning vector. The linker may be located either upstream or downstream of the therapeutic protein, and it may be located either upstream or downstream of the nucleic acid encoding the fusion partner.

Alternatively, a linker may be a chemical structure or reagent that attaches chemical moieties, e.g., polymers, polyamino acids or branch point amino acids to a polypeptide of the invention.

[045] A "vector" is used to transfer nucleic acid sequences from one organism to another. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. Expression vectors can be used to express the polypeptides of the invention and typically include restriction sites to provide for the insertion of nucleic acid sequences encoding heterologous protein or RNA molecules.

[046] A "polymerase chain reaction" is a chemical reaction capable of amplifying DNA *in vitro*.

[047] A "regulatory sequence" is a nucleic acid sequence involved in regulating the expression, e.g., transcription, of other genes. Regulatory sequences include, but are not limited to, promoters, enhancers, initiation and termination regions, other control regions, expression regulatory factors, and expression controls.

[048] An "expression system" is a combination of an expression vector, its cloned DNA, and the host for the vector that provide a context in which foreign genes can function, e.g., to produce proteins, e.g., in a host cell. An expression vector is a cloning vector that contains regulatory sequences that allow transcription of a cloned gene or genes and thus express cloned DNA. An expression system can perform a process by which a gene's coded information is converted into the molecules present and operating in the cell, e.g., RNA and polypeptides.

[049] The term "host cell" includes an individual cell, cell line, cell culture, or *in vivo* cell, which can be or has been a recipient of any polynucleotide or polypeptide of the invention, for example, a recombinant vector, an isolated polynucleotide, antibody, or fusion protein.

[050] A "therapeutic molecule" is one that is palliative, curative, or otherwise useful in treating or ameliorating a disease, disorder, syndrome, or condition. A "prophylactic molecule" is one that prevents the occurrence or recurrence of a disease, disorder, syndrome, or condition, e.g., a drug or vaccine.

[051] A "cytokine" is a small, soluble protein secreted by a cell. It affects, e.g., interactions between cells, communications between cells or the behavior of cells. Cytokines include, but are not limited to, the interleukins, lymphokines, chemokines, cell signalling molecules, and interferons.

[052] "Plasma stability" refers to the tendency of a molecule to retain its biological activity in plasma *in vivo*. It can be determined, e.g., by the molecule's bodily absorption, distribution, metabolism, and excretion.

[053] A "pharmaceutically acceptable carrier" refers to a non-toxic solid, semisolid, or liquid filler, diluent, encapsulating material, or formulation auxiliary of any conventional type. "Carrier" and "excipient" are used interchangeably herein. A pharmaceutically acceptable carrier is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the carrier for a formulation containing polypeptides does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

[054] A "buffer" is a system that tends to resist change in pH when a given increment of hydrogen ion or hydroxide ion is added. At pH values outside the buffer zone there is less capacity to resist changes in pH. The buffering power is maximal at the pH where the concentration of the proton donor (acid) equals that of the proton acceptor (base). Buffered solutions contain conjugate acid-base pairs. A buffered solution will demonstrate a lesser change

in pH than an unbuffered solution in response to addition of an acid or base. Any conventional buffer can be used with the compositions herein including but not limited to, for example, Tris, phosphate, imidazole, and bicarbonate.

B. FETUIN FUSION MOLECULES

1. FETUIN FUSION PARTNERS

[055] The invention relates to a recombinant fusion polypeptide composed of fetuin or a variant of fetuin coupled to an active polypeptide derived from a natural or an artificial polypeptide with biological activity. The natural fetuin polypeptides of the invention encompass all known isoforms and splice variants of fetuin A and B. Polypeptides of the invention include full length proteins and fragments thereof, particularly biologically active fragments and/or fragments corresponding to functional domains, e.g., a signal peptide or leader sequence, an enzyme active site, including a cleavage site and an enzyme catalytic site, a domain for interaction with other protein(s), a domain for binding DNA, a regulatory domain, a consensus domain that is shared with other members of the same protein family, such as a cytokine family; an extracellular domain that may act as a target for antibody production or that may be cleaved to become a soluble receptor or a ligand for a receptor; an intracellular fragment of a transmembrane protein that participates in signal transduction; a transmembrane domain of a transmembrane protein that may facilitate water or ion transport; a sequence associated with cell survival and/or cell proliferation; a sequence associated with cell cycle arrest, DNA repair and/or apoptosis; a sequence associated with a disease or disease prognosis, including types of cancer, degenerative disease, inflammatory disease, immunological disease, genetic disease, metabolic disease, and/or bacterial or viral infection; and including fusions of the subject polypeptides to other proteins or parts thereof; modifications of the subject polypeptide, e.g., comprising

modified, derivatized, or designer amino acids, modified peptide backbones, and/or immunological tags; as well as intra- and inter-species homologs of the subject polypeptides.

[056] The fetuin variants of the invention encompass any fetuin polypeptide with a high plasma half-life which is obtained by modification, such as by mutation, deletion, or addition. The invention encompasses all fetuin variants with a high plasma half-life obtained by *in vitro* modification of a polypeptide encoded by a fetuin polynucleotide. It includes non-natural sequences isolated from random peptide libraries. It also includes natural or artificial post-translational modifications, such as prenylation, glycosylation, e.g., with sialic acid, and the like. Modifications can be performed by any technique known in the art, such as commonly employed genetic engineering techniques. Such modified polypeptides can show, e.g., enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

[057] For instance, for many proteins, including the extracellular domain of a membrane associated protein or the mature form(s) of a secreted protein, it is known in the art that one or more amino acids may be deleted from the N-terminus or C-terminus without substantial loss of biological function. For instance, Ron et al., 1993, reported modified keratinocyte growth factor proteins that retained heparin binding activity even when 3, 8, or 27 N-terminal amino acid residues were missing.

[058] However, even if deletion of one or more amino acids from the N-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein will

generally be retained when fewer than the majority of the residues of the complete or mature protein are removed from the N-terminus. Whether a particular polypeptide lacking N-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

[059] Similarly, many examples of biologically functional C-terminal deletion mutants are known. For instance, interferon gamma shows as much as a ten-fold increase in biological activity when 8-10 amino acid residues are deleted from the carboxy terminus of the protein (Dobeli et al., 1988). However, even if deletion of one or more amino acids from the C-terminus of a protein results in modification or loss of one or more biological functions of the protein, other biological activities may still be retained. Thus, the ability of the shortened protein to induce and/or bind to antibodies which recognize the complete or mature form of the protein will generally be retained when fewer than the majority of the residues of the complete or mature protein are removed from the C-terminus. Whether a particular polypeptide lacking C-terminal residues of a complete protein retains such immunologic activities can readily be determined by routine methods described herein and otherwise known in the art.

[060] In addition to terminal deletion forms of the protein discussed above, it also will be recognized by one of ordinary skill in the art that some amino acid sequences of the herein described fetuin polypeptides can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas of the protein which determine the plasma stability.

[061] Thus, the invention further includes variations of the present fetuin polypeptides that demonstrate a half-life about as long, or longer, as the unmodified protein, or which include regions of the fetuin protein such as the protein portions discussed below. Such variations

include deletions, insertions, inversions, repeats, and type substitutions selected according to general rules known in the art so as to have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al., 1990, wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene followed by selection or screening to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are tolerant of amino acid substitutions. The authors further indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie, et al., *supra*, and the references cited therein. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between the amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among the aromatic residues Phe and Tyr.

[062] Thus, a fragment, derivative or analog of fetuin may be one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code; one in which one or more of the amino acid residues includes a substituent group; one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example,

polyethylene glycol); one in which the additional amino acids are fused to the above form of the polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence, or a sequence which is employed for purification of the above form of the polypeptide; or a proprotein sequence. Such fragments, derivatives, and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

[063] Amino acid residues in the fetuin polypeptides of the present invention that are determinative of the *in vivo* half-life can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, 1989). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules can then be tested for biological activity such as receptor binding, or *in vivo* or *in vitro* proliferative activity.

[064] The invention provides an antibody, which is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen; antibodies are commonly known in the art. The invention further provides for an epitope, which is the site of an antigenic molecule to which an antibody binds.

[065] Also provided by the invention is a peptide or polypeptide comprising an epitope-bearing portion of either the fetuin fusion partner or the therapeutic fusion partner. The epitope of this polypeptide portion can be an immunogenic epitope, i.e., a part of a protein that elicits an antibody response when the whole protein is the immunogen, or an antigenic epitope, i.e., a region of a protein molecule to which an antibody can bind. The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes (Geysen et al., 1983).

[066] The fetuin molecules of the present invention may prepared using glycosylated, non-glycosylated, or de-glycosylated fetuin and variants of fetuin. Suitable chemical moieties for derivatization of fetuin and variants of fetuin include, for example, polymers, such as the water soluble polymers described herein.

[067] A modified fetuin polypeptide of the invention can be prepared by attaching polyamino acids or branch point amino acids to the fetuin polypeptide. For example, the polyamino acid may be a carrier protein that serves to increase the circulation half life of the fetuin polypeptide (i.e., in addition to the advantages achieved via a fetuin fusion molecule). For the therapeutic purpose of the present invention, such polyamino acids should ideally be those that have or do not create a neutralizing antigenic response, or other adverse responses. Such polyamino acids may include serum album (such as human serum albumin), an additional antibody or portion thereof, for example the Fc region, or other polyamino acids, e.g. lysines. As described herein, the location of attachment of the polyamino acid may be at the N-terminus, or C-terminus, or other places in between, and also may be connected by a chemical "linker" moiety to the selected fetuin molecule.

[068] Polymers, and in particular water soluble polymers, are useful in the present invention, as the polypeptide to which each polymer is attached will not precipitate in an aqueous environment, such as a physiological environment. Preferably, polymers employed in the invention will be pharmaceutically acceptable for the preparation of a therapeutic product or composition. One skilled in the art will be able to select the desired polymer based on such considerations as the desired dosage, circulation time, and resistance to proteolysis.

[069] Suitable, clinically acceptable, water soluble polymers include, but are not limited to, polyethylene glycol (PEG), polyethylene glycol propionaldehyde, copolymers of ethylene

glycol/propylene glycol, monomethoxy-polyethylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol (PVA), polyvinyl pyrrolidone, poly-1,3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, poly (β-amino acids) (either homopolymers or random copolymers), poly(n-vinyl pyrrolidone) polyethylene glycol, polypropylene glycol homopolymers (PPG) and other polyakylene oxides, polypropylene oxide/ethylene oxide copolymers, polyoxyethylated polyols (POG) (e.g., glycerol) and other polyoxyethylated polyols, polyoxyethylated sorbitol, or polyoxyethylated glucose, colonic acids or other carbohydrate polymers, Ficoll or dextran, and mixtures thereof. PEG can encompass any of the forms that have been used to derivatize other proteins, such as mono-(C1-C10) alkoxy- or aryloxy-polyethylene glycol. Polyethylene glycol propionaldehyde may have advantages in manufacturing due to its stability in water.

[070] A modified fetuin polypeptide of the invention can also be prepared by attaching polymers to the fetuin polypeptide. Polymers used herein, may be of any molecular weight and may be branched or unbranched. The polymers each typically have an average molecular weight of between about 2 kDa to about 100 kDa. The average molecular weight of each polymer is preferably between about 5 kDa and about 50 kDa, more preferably between about 12 kDa and about 25 kDa. Generally, the higher the molecular weight or the more branches, the higher the polymer:protein ratio. Other sizes may be used, depending on the desired therapeutic profile, for example, the duration of sustained release; the effects, if any, on biological activity; the ease in handling; the degree or lack of antigenicity; and other known effects of a polymer on a fetuin molecule.

[071] Polymers employed in the present invention are typically attached to a fetuin molecule with consideration of their effects on functional or antigenic domains of the

polypeptide. In general, chemical derivatization may be performed under any suitable condition used to react a protein with an activated polymer molecule. Activating groups which can be used to link the polymer to the active moieties include sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane, and 5-pyridyl.

[072] Polymers of the invention are typically attached to a fetuin polypeptide of the invention at the alpha (α) or epsilon (ε) amino groups of amino acids or a reactive thiol group, but it is also contemplated that a polymer group could be attached to any reactive group of the protein that is sufficiently reactive to become attached to a polymer group under suitable reaction conditions. Thus, a polymer may be covalently bound to a fetuin polypeptide of the invention via a reactive group, such as a free amino or carboxyl group. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residue. Those having a free carboxyl group may include aspartic acid residues, glutamic acid residues, and the C-terminal amino acid residue. Those having a reactive thiol group include cysteine residues.

[073] Methods for preparing fetuin fusion molecules conjugated with polymers, such as water soluble polymers, will each generally involve reacting a protein with a polymer under conditions whereby the protein becomes attached to one or more polymers, and obtaining the reaction product. Reaction conditions for each conjugation may be selected from any of those known in the art or those subsequently developed, but should be selected to avoid or limit exposure to reaction conditions such as temperatures, solvents, and pH levels that would inactivate the protein to be modified. In general, the optimal reaction conditions for the reactions will be determined case-by-case based on known parameters and the desired result. For example, the larger the ratio of polymer:protein conjugate, the greater the percentage of conjugated product. The optimum ratio, in terms of efficiency of the reaction so that there is no

excess unreacted protein or polymer, may be determined by factors such as the desired degree of derivatization (e.g., mono-, di-tri- etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions used. The ratio of polymer (e.g., PEG) to fetuin polypeptide will generally range from 1:1 to 100:1. One or more purified conjugates may be prepared from each mixture by standard purification techniques, including among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography, and electrophoresis.

[074] In an embodiment, the present invention contemplates the chemically derivatized fetuin polypeptide to include mono- or poly- (e.g., 2-4) PEG moieties. "Pegylation" may be carried out by any of the pegylation reactions known in the art. Methods for preparing a pegylated protein product will generally include reacting a polyepeptide with polyethylene glycol (such as a reactive ester or aldehyde derivative of PEG) under conditions whereby the protein becomes attached to one or more PEG groups, and obtaining the reaction product(s). In general, the optimal reaction conditions for the reactions will be determined case by case based on known parameters and the desired result. There are a number of PEG attachment methods available to those skilled in the art, e.g., EP 0 401 384; Malik et al., 1992; Francis et al., 1992; EP 0 154 316; EP 0 401 384; WO 92/16221; WO 95/34326; and other publications cited herein that relate to pegylation, the disclosures of which are hereby incorporated by reference.

[075] The step of pegylation, as described herein, may be carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule. Thus, protein products according to the present invention include pegylated proteins wherein one or more PEG groups are attached via acyl or alkyl groups. The PEG groups are generally attached to the protein at the α- or ε-amino groups of amino acids, but it is also contemplated that the PEG

groups could be attached to any amino group attached to the protein that is sufficiently reactive to become attached to a PEG group under suitable reaction conditions.

[076] For acylation reactions, the polymer(s) selected typically have a single reactive ester group. Any known or subsequently discovered reactive PEG molecule may be used to carry out the pegylation reaction. A preferred activated PEG ester is PEG esterified to N-hydroxysuccinimide (NHS). As used herein, "acylation" is contemplated to include, without limitation, the following types of linkages between fetuin and a polymer such as PEG, for example, an amide, carbamate, or urethane (Chamow et al., 1994). Reaction conditions may be selected from any of those known in the pegylation art or those subsequently developed, but should avoid conditions such as temperature, solvent, and pH that would inactivate the polypeptide to be modified.

[077] Pegylation by acylation will generally result in a poly-pegylated protein. Preferably, the connecting linkage will be an amide. Also preferably, the resulting product will be substantially only (e.g., >95%) mono, di- or tri-pegylated. However, some species with higher degrees of pegylation may be formed in amounts depending on the specific reaction conditions used. If desired, more purified pegylated species may be separated from the mixture (particularly unreacted species) by standard purification techniques, including among others, dialysis, salting-out, ultrafiltration, ion-exchange chromatography, gel filtration chromatography and electrophoresis.

[078] Pegylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with the protein in the presence of a reducing agent. For the reductive alkylation reaction, the polymer(s) selected should have a single reactive aldehyde group. An

exemplary reactive PEG aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof, see for example, U.S. Pat. No. 5,252,714.

[079] One may specifically desire an N-terminal chemically modified protein. One may select a polymer by molecular weight, branching, etc., the proportion of polymers to protein (or peptide) molecules in the reaction mix, the type of reaction to be performed, and the method of obtaining the selected N-terminal chemically modified protein. The method of obtaining the N-terminal chemically modified protein preparation (i.e., separating this moiety from other monoderivatized moieties if necessary) may be by purification of the N-terminal chemically modified protein material from a population of chemically modified protein molecules.

[080] Selective N-terminal chemical modification may be accomplished by reductive alkylation which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminus) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a polymer containing a carbonyl group is achieved. For example, one may selectively attach a polymer to the N-terminus of the protein by performing the reaction at a pH which allows one to take advantage of the pKa differences between the ε-amino group of the lysine residues and the α-amino group of the N-terminal residue of the protein. By such selective derivatization, attachment of a polymer to a protein is controlled; the conjugation with the polymer takes place predominantly at the N-terminus of the protein and no significant modification of other reactive groups, such as the lysine side chain amino groups, occurs. Using reductive alkylation, the polymer may be of the type described above and should have a single reactive aldehyde for coupling to the protein. Polyethylene glycol propionaldehyde, containing a single reactive aldehyde, may also be used.

2. THERAPEUTIC FUSION PARTNERS

[081] The therapeutic components of the fusion molecule can be derived from human or non-human origins. They may be above-mentioned cytokines, or any other biologically active peptide, or derivative thereof. For example, the therapeutic molecule or prophylactic molecule may be all or part of an enzyme, an enzyme inhibitor, a kinase, a phosphatase, an antigen, an antibody, a hormone, a receptor, a ligand, a factor involved in cell motility or migration, a factor involved in cell division or replication, a factor involved in interaction with the extracellular matrix, and/or a factor involved in apoptosis.

[082] The therapeutic fusion partner may comprise all or part of a detectable protein, e.g., an epitope tag, such as hemagglutinin, FLAG, or c-myc; polypeptides that provide a detectable signal or that serve as detectable markers, e.g., a fluorescent protein, such as green fluorescent protein, a fluorescent protein from an Anthozoan species; β-galactosidase; luciferase; or cre recombinase; polypeptides that provide a catalytic function or induce a cellular response; polypeptides that provide for secretion of the fusion protein from a eukaryotic cell; polypeptides that provide for secretion of the fusion protein from a prokaryotic cell; polypeptides that provide for binding to metal ions (e.g., His_n, where n = 3-10, e.g., 6His); and structural proteins. Fusion partners can also be those that are able to stabilize the therapeutic polypeptide, such as polyethylene glycol (PEG), or a fragment of an immunoglobulin, such as the Fc fragment of IgG, IgE, IgA, IgM, and/or IgD.

3. *IN VIVO* PLASMA STABILITY

[083] The invention provides a high plasma stability for the polypeptide of the invention. The fusion molecule of the invention has a higher plasma stability than an unfused therapeutic molecule or prophylactic molecule. The stability of the fusion molecules of the invention makes it possible to maintain the biological activity of the active component for a

prolonged period *in vivo*. This in turn makes it possible to reduce the frequency and the strength of the effective dosage. In some cases, this can increase the therapeutic effect, e.g., by reducing the incidence or severity of side effects that follow a higher dosage regimen.

[084] Modification of asialofetuin (ASF) with NN-dilactitol-N'-fluoresceinylethylethylenediamine (DLF) reportedly affected neither its normal kinetics of clearance from the circulation nor its normal tissue sites of uptake and degradation (Maxwell et al., 1990). After injection of DLF-ASF, fluorescent degradation products were retained with a half-life of about 2 days (Maxwell et al., 1990). ASF is degraded by the liver, with a half-life of about two days, whether it is unconjugated or conjugated (Wu et al., 1998). Thus, fusion proteins of fetuin and a therapeutic protein of interest will possess a half life of about two days. This represents a considerable improvement for many proteins with proven or hypothesized therapeutic value.

[085] Detection methods of the fusion molecule *in vivo* are chosen based on the detectable fusion partner. For example, where the fusion partner provides an immunologically recognizable epitope, an epitope-specific antibody can be used to quantitatively detect the level of polypeptide. In some embodiments, the fusion partner provides a detectable signal, and in these embodiments, the detection method is chosen based on the type of signal generated by the fusion partner. For example, where the fusion partner is a fluorescent protein, fluorescence is measured.

[086] Where the fusion partner is an enzyme that yields a detectable product, the product can be detected using an appropriate means. For example, β-galactosidase can, depending on the substrate, yield a colored product that can be detected with a

spectrophotometer, and the fluorescent protein luciferase can yield a luminescent product detectable with a luminometer.

C. CONSTRUCTION OF FETUIN FUSION MOLECULES

[087] The present invention also relates to vectors which include the isolated nucleic acid molecules of the present invention and host cells which are genetically engineered with the recombinant vectors. These vectors and host cells can be used for the production of fetuin polypeptides described herein, including fragments thereof produced by conventional recombinant techniques. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. A plasmid is a small, independently replicating piece of extrachromosomal cytoplasmic DNA that can be transferred from one organism to another. Plasmids can become incorporated into the genome of a host or may remain independent. Artificially constructed plasmids are commonly used as cloning vectors. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

[088] Polynucleotides of the invention include single-stranded, double-stranded and triple helical molecules. Polynucleotides may also be oligonucleotides which generally refer to polynucleotides of between about 5 and about 100 nucleotides of single- or double-stranded DNA or RNA. For the purposes of this disclosure, there is no upper limit to the length of an oligonucleotide. Oligonucleotides are also known as oligomers or oligos and can be isolated from genes, or chemically synthesized by methods known in the art.

[089] Non-limiting embodiments of polynucleotides include genes or gene fragments, exons, introns, mRNA, tRNA, rRNA, siRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of

any sequence, nucleic acid probes, and primers. Polynucleotides include splice variants of an mRNA. Nucleic acids can be naturally occurring, e.g. DNA or RNA, or can be synthetic analogs, as known in the art. Such analogs are suitable as probes because they demonstrate stability under assay conditions. A nucleic acid molecule can also comprise modified nucleic acid molecules, such as methylated nucleic acid molecules and nucleic acid molecule analogs. Analogs of purines and pyrimidines are known in the art.

[090] Nucleic acid compositions can comprise a sequence of DNA or RNA, including one having an open reading frame that encodes a polypeptide and is capable, under appropriate conditions, of being expressed as a polypeptide. Expression of a nucleic acid molecule refers to the conversion of the information into a gene product. A gene product can be the direct transcriptional product of a gene (e.g., mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA, or any other type of RNA) or a protein produced by translation of an mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[091] The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

[092] The nucleic acid insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp, phoA, and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs. Other suitable promoters will be

known to the skilled artisan. A promoter is a nucleotide sequence present in DNA, to which RNA polymerase binds to begin transcription. The term includes a DNA regulatory region capable of binding RNA polymerase in a mammalian cell and initiating transcription of a downstream (3' direction) coding sequence operably linked thereto. For purposes of the present invention, a promoter sequence includes the minimum number of bases or elements necessary to initiate transcription of a gene of interest at levels detectable above background. Within the promoter sequence is a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

[093] Heterologous promoters are derived from different genetic sources. They encompass promoters of different species, e.g., a rat promoter is heterologous to a human promoter of the corresponding gene. The term also includes promoters found in different cell or tissue types of a specimen of the same species, e.g., a promoter active in the transcription of a protein in human brain may be heterologous to a promoter active in the transcription of the same protein in human muscle. Heterologous promoters can be natural or artificial, and comprised of different elements. A promoter that naturally regulates is one that regulates in nature and without artificial aid. The term can include heterologous and homologous promoters. A tissue specific promoter is one that initiates transcription exclusively or selectively in one or a few tissue types.

[094] The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation

initiating codon at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[095] As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture, and tetracycline, kanamycin, or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces*, and *Salmonella typhimurium* cells; fungal cells, such as yeast cells, including *Saccharomyces* and *Pichia* cells; insect cells, such as Drosophila S2 and Spodoptera Sf9 cells; animal cells, such as CHO, COS, 293, and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

[096] Among vectors preferred for use in bacteria include pQE70, pQE60, and pQE-9, available from QIAGEN, Inc., (Valencia, CA); pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH6a, pNH18A, pNH46A, available from Stratagene (La Jolla, CA); and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG, and pSVL available from Pharmacia (Kalamazoo, MI). Other suitable vectors will be readily apparent to the skilled artisan.

[097] Fetuin fusion molecules may be constructed using the polymerase chain reaction. The reaction is performed *in vitro* using two oligonucleotide primers, which are complementary to two regions of the target DNA to be amplified, one for each strand. A primer is a polynucleotide chain to which deoxyribonucleotides can be added by DNA polymerase. The primers are added to the target DNA in the presence of excess deoxynucleotides and a heat stable

DNA polymerase. The target DNA can be provided to the reaction mixture in pure or relatively pure form, or it may be present as a minor component, as is typically the case when it is provided as a component of a biological sample. In a series of temperature cycles, the target DNA is repeatedly denatured at high temperature, annealed to the primer at a lower temperature, and a daughter strand extended from the primer at an intermediate temperature. As the daughter strands act as templates in subsequent temperature cycles, DNA fragments matching both primers are amplified exponentially.

[098] Introduction of the construct into a host cell can be effected by a variety of methods such as transformation, transfection and transduction. When a host cell is transformed, it is subjected to a process by which the genetic material carried by an individual cell is altered by incorporation of exogenous DNA into its genome. Transfection of a host cell is the introduction of a nucleic acid into a recipient cell and the subsequent integration into the chromosomal DNA of the recipient cells. Transduction of a host cell is the transfer of genetic information from one cell to another via a vector. These processes may be carried out by calcium phosphate mediated transfection, DEAE-dextran mediated transfection, cationic lipid mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., 1995.

[099] A host cell of the invention includes an individual cell, cell line, cell culture, or *in vivo* cell, which can be or has been a recipient of any polynucleotide or polypeptide of the invention, for example, a recombinant vector, an isolated polynucleotide, antibody, or fusion protein. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology, physiology, or in total DNA, RNA, or polypeptide complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or

change. Host cells can be prokaryotic or eukaryotic, including mammalian, insect, amphibian, reptile, crustacean, avian, fish, plant, and fungal cells. A host cell includes cells transformed, transfected, transduced, or infected *in vivo* or *in vitro* with a polynucleotide of the invention, for example, a recombinant vector. A host cell which comprises a recombinant vector of the invention may be called a recombinant host cell. After introducing the vector or DNA construct encoding polypeptides of the invention into host cells, by any of the methods described above, the cells can be allowed to grow to produce the polypeptides.

[0100] The presently described polypeptides, whether modified or unmodified, may include secretion signals, and also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide.

[0101] The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability, and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to stabilize and purify proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins containing various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after

the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists (Bennett et al., 1995; Johanson et al., 1995).

[0102] A fetuin fusion molecule of the invention can be recovered and purified from recombinant cell cultures by well-known methods, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Most preferably, high performance liquid chromatography (HPLC) is employed for purification. Polypeptides of the present invention include products purified from natural sources, including bodily fluids, tissues, and cells, whether directly isolated or cultured; products of chemical synthetic procedures; and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect, and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes. Thus, it is well known in the art that the N-terminal methionine encoded by the translation initiation codon generally is removed with high efficiency from any protein after translation in all eukaryotic cells. While the N-terminal methionine on most proteins also is efficiently removed in most prokaryotes, for some proteins this prokaryotic removal process is

inefficient, depending on the nature of the amino acid to which the N-terminal methionine is covalently linked.

D. THERAPEUTIC APPLICATIONS

1. COMPOSITIONS

[0103] The invention provides recombinant polypeptides which are biologically active and can be used pharmaceutically. The therapeutic activity of the polypeptides of the invention can be used to treat disease, or can be used indirectly, for example, by designing vaccines, in medical research, and the like. The polypeptides of the invention can also be directed toward prophylaxis, or preventing disease, and towards diagnosing disease.

[0104] As encompassed by the invention, treatment refers to any administration or application of remedies for disease in an animal, including a human, and includes inhibiting the disease, i.e., arresting its development, or relieving the disease, i.e., causing its regression; or restoring or repairing a lost, missing, or defective function; or stimulating an inefficient process. A disease is a pathological, abnormal, and/or harmful condition of an organism and includes conditions, syndromes, and disorders. Prophylaxis includes preventing a disease from occurring or recurring in a subject that may be predisposed to the disease but has not yet been diagnosed as having it. Treatment and prophylaxis can be administered to an organism, or to a cell *in vivo, in vitro,* or *ex vivo*, and the cell subsequently administered to the patient, subject, or individual, which is a mammal. Representative examples of a patient, subject, or individual include, but are not limited to, humans, murines, simians, felines, canines, equines, bovines, porcines, ovines, caprines, avians, mammalian farm animals, mammalian sport animals, and mammalian pets.

[0105] The invention provides compositions containing an effective amount of a fetuin fusion molecule and a pharmaceutically acceptable carrier or vehicle. The compositions are suitable for veterinary or human administration. The compositions can be provided be in any

form that allows for the composition to be administered to an animal. For example, the composition can be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral, sublingual, rectal, vaginal, ocular, and intranasal. Parenteral administration includes subcutaneous injections, intravenous, intramuscular, intrasternal injection, or infusion techniques. Preferably, the compositions are administered parenterally. Pharmaceutical compositions of the invention can be formulated so as to allow a molecule of the invention to be bioavailable upon administration of the composition to an animal. Compositions can take the form of one or more dosage units, where for example, a tablet can be a single dosage unit, and a container of a molecule of the invention in aerosol form can hold a plurality of dosage units.

[0106] Materials used in preparing the pharmaceutical compositions can be non-toxic in the amounts used. It will be evident to those of ordinary skill in the art that the optimal dosage of the active ingredient(s) in the pharmaceutical composition will depend on a variety of factors. Relevant factors include, without limitation, the type of animal (e.g., human), the particular form of the molecule of the invention, the manner of administration, and the composition employed.

[0107] The pharmaceutically acceptable carrier or vehicle can be particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) can be liquid, with the compositions being, for example, an oral syrup or injectable liquid. In addition, the carrier(s) can be gaseous, so as to provide an aerosol composition useful in, e.g., inhalatory administration.

[0108] When intended for oral administration, the composition is preferably in solid or liquid form, where semi-solid, semi-liquid, suspension, and gel forms are included within the forms considered herein as either solid or liquid. As a solid composition for oral administration, the composition can be formulated into a powder, granule, compressed tablet, pill, capsule,

chewing gum, wafer, or a like form. Such a solid composition typically contains one or more inert diluents. In addition, one or more binders, such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, or gelatin; excipients such as starch, lactose or dextrins; or disintegrating agents, such as alginic acid; or sodium may be present.

[0109] Suitable carriers include, but are not limited to, water, dextrose, glycerol, saline, ethanol, and combinations thereof. The carrier can contain additional agents such as wetting or emulsifying agents, pH buffering agents, or adjuvants, which enhance the effectiveness of the formulation. Topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolaurate (5%) in water, or sodium lauryl sulfate (5%) in water.

[0110] Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents can be added as necessary. Percutaneous penetration enhancers such as Azone can also be included. Compositions for oral administration can form solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders.

2. FORMULATIONS

[0111] The fetuin polypeptide composition will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual subject, the site of delivery of the fetuin polypeptide composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" of fetuin polypeptide for purposes herein is thus determined by such considerations.

[0112] The polypeptides of the present invention may be employed in combination with a suitable pharmaceutical carrier to comprise a pharmaceutical composition for parenteral administration. Such compositions comprise a therapeutically effective amount of the polypeptide and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is

not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

[0113] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the fetuin fusion molecules of the invention may be employed in conjunction with other therapeutic compounds.

[0114] The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10 microg/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day.

[0115] Polynucleotides encoding fetuin fusion polypeptides can be used in gene therapy to transiently or permanently alter the cellular phenotype of patients in need of such treatment (Bast et al., 2000). Gene therapy can suppress the disease phenotype, e.g., by down-regulating genes that contribute to disease progression, by reversing the transformed phenotype, and/or by inducing cell death. Gene therapy can also result in the production of the therapeutic molecule or prophylactic molecule, e.g., the fetuin fusion molecule, *in vivo*. Such *in vivo* production may have systemic effects resulting in the treatment of disease. Gene therapy can be performed *in vivo* or *ex vivo*. *In vivo* gene therapy can be accomplished by directly administering the gene

therapeutic to a patient. The patient's cells are transfected or transduced with a vector containing the polynucleotide encoding a fetuin fusion molecule. *Ex vivo* gene therapy can be accomplished by transfecting or transducing a vector containing the polynucleotide encoding a fetuin fusion molecule into cells *in vitro* and then administering them to the patient. Such cells may be either autologous or allogeneic. Transfection of a fetuin fusion molecule involves its direct introduction into the cell. Transduction of a fetuin fusion molecule involves its introduction into the cell via a vector.

[0116] Both viral and non-viral vectors are suitable for use in *in vivo* or *ex vivo* gene therapy. Suitable viral vectors include retroviruses, adenoviruses, herpes viruses, and adeno-associated viruses. Viral vectors can enter cells by receptor-mediated processes and deliver nucleic acids to the cell interior. Non-viral delivery systems suitable for the invention include those useful for transfecting plasmids into cells, e.g., calcium phosphate precipitation, electroporation, or liposomes. The compositions of the invention may also be introduced into the target cell by microinjection. They may be introduced into target cells by vesicle fusion, e.g., fusion of cationic liposomes with the plasma membrane. They may be directly injected into a target tissue. Direct injection techniques include particle-mediated nucleic acid transfer by physical force, i.e., by a particle bombardment device, or "gene gun" (Tang et al., 1992), by coating microprojectiles with nucleic acids, then bombarding them into cells. The microprojectiles may, e.g., be gold particles, and injection can be used to deliver the fetuin fusion protein intradermally, intramuscularly, or into other tissues, as needed (Furth et al., 1992). Viral or non-viral fetuin fusion molecule vectors can be used in both *in vivo* or *ex vivo* gene therapy.

[0117] The invention also provides for the administration of a nucleic acid vaccine by providing an effective amount of the fetuin fusion molecules to a patient. Administration of a

vaccine of the invention can lead to the persistent expression and release of the therapeutic or prophylactic immunogen over a period of time. These vaccines may induce humoral responses. They may also induce cellular responses, for example, by stimulating T-cells that recognize and kill cells, e.g., tumor cells, directly. For example, nucleotide-based vaccines of the invention encoding tumor antigens can be used to activate the $CD8^+$ cytotoxic T lymphocyte arm of the immune system. In some embodiments, the vaccines activate T-cells directly, and in others they enlist antigen-presenting cells to activate T-cells. Killer T-cells are primed, in part, by interacting with antigen-presenting cells, i.e., dendritic cells. In some embodiments, plasmids comprising the nucleic acid molecules of the invention enter antigen-presenting cells, which in turn display the encoded tumor-antigens that contribute to killer T-cell activation. Again, the tumor antigens can be delivered as plasmid DNA constructs, or introduced via viral or non-viral vectors, either alone or with other molecules. Also by way of example, dendritic cells can be transfected with mRNA encoding tumor antigens (Heiser et al., 2002; Mitchell and Nair, 2000).

[0118] Similarly, cells may be engineered *in vivo* for expression of the polypeptide *in vivo*, for example, by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such methods should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retroviral particle, for example, an adenovirus, adeno-associated virus, or herpesvirus, which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

[0119] Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

[0120] Vectors suitable for use in these formulations include one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter (Miller and Rosman, 1989), or any other promoter, for example, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters. Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

[0121] Suitable promoters may also include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAl promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the beta-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

[0122] The retroviral plasmid vector is employed to transfect packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, -2, -AM, PA12, T19-14X, VT-19-17-H2, CRE, CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, 1990. The vector may be introduced into the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and calcium phosphate precipitation. In an embodiment, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

[0123] The producer cell line generates infectious, replication incompetent retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

E. EMBODIMENTS OF THE INVENTION

[0124] In accordance with an objective of the present invention, there is provided:

1. A fusion molecule comprising a first polypeptide that comprises a first amino acid sequence of a therapeutic molecule or a prophylactic molecule and a second polypeptide that comprises a second amino acid sequence of a fusion partner, wherein the fusion partner is a fetuin polypeptide.

2. The fusion molecule of 1, wherein the fusion molecule has a higher plasma stability than the therapeutic molecule or prophylactic molecule absent the fusion partner.

3. The fusion molecule of 1, wherein the fetuin polypeptide is at least a fragment of fetuin A polypeptide.

4. The fusion molecule of 1, wherein the fetuin polypeptide is at least a fragment of human fetuin A polypeptide.

5. The fusion molecule of 1, wherein the fetuin polypeptide is at least a fragment of fetuin B polypeptide.

6. The fusion molecule of 1, wherein the fetuin polypeptide is at least a fragment of human fetuin B polypeptide.

7. The fusion molecule of 1, wherein the fetuin polypeptide is a variant of fetuin A or fetuin B.

8. The fusion molecule of 1, wherein the fetuin polypeptide is a variant of human fetuin A or human fetuin B.

9. The fusion molecule of 7 or 8, wherein the variant comprises a mutation of one or more amino acid residues.

10. The fusion molecule of 7 or 8, wherein the variant comprises a deletion of one or more amino acid residues.

11. The fusion molecule of 10, wherein the deletion comprises a deletion of amino acid residue 71 of human fetuin A.

12. The fusion molecule of 10, wherein the deletion comprises a deletion of amino acid residues 226 to 253 of human fetuin A.

13. The fusion molecule of 7 or 8, wherein the variant comprises a mutation of one or more amino acid residues and a deletion of one or more amino acid residues.

14. The fusion molecule of 7 or 8, wherein the variant comprises an addition of one or more amino acid residues.

15. The fusion molecule of 7 or 8, wherein the variant comprises a mutation of one or more amino acid residues and an addition of one or more amino acid residues.

16. The fusion molecule of 1, wherein the fetuin polypeptide comprises a mature polypeptide.

17. The fusion molecule of 1, wherein the fusion molecule comprises a linker.

18. The fusion molecule of 17, wherein the linker is a peptide linker.

19. The fusion molecule of 1 or 18, wherein the fusion partner is linked to the N-terminus of the therapeutic molecule or prophylactic molecule.

20. The fusion molecule of 1 or 18, wherein the fusion partner is linked to the C-terminus of the therapeutic molecule or prophylactic molecule.

21. The fusion molecule of 18, wherein the peptide linker comprises an amino acid sequence that is present in a precursor fetuin polypeptide.

22. The fusion molecule of 17, wherein the linker comprises an enzyme cleavage site.

23. The fusion molecule of 1, wherein the fetuin polypeptide comprises a sequence of at least six consecutive amino acid residues chosen from a naturally occurring fetuin polypeptide.

24. The fusion molecule of 1, wherein the fetuin polypeptide comprises an N-terminal amino acid sequence of a mature polypeptide of a naturally occurring fetuin.

25. The fusion molecule of 24, wherein the mature polypeptide of a naturally occurring fetuin is human fetuin A or human fetuin B.

26. The fusion molecule of 25, wherein the N-terminal amino acid sequence comprises at least six consecutive amino acid residues chosen from amino acid residues 23-132 of human fetuin A and amino acid residues 37-104 of human fetuin B.

27. The fusion molecule of 25, wherein the N-terminal amino acid sequence comprises at least six consecutive amino acid residues chosen from amino acid residues 23-247 of human fetuin A and amino acid residues 37-254 of human fetuin B.

28. The fusion molecule of 1, wherein the fetuin polypeptide comprises a C-terminal amino acid sequence of a mature polypeptide of a naturally occurring fetuin.

29. The fusion molecule of 28, wherein the mature polypeptide of a naturally occurring fetuin is human fetuin A or human fetuin B.

30. The fusion molecule of 29, wherein the C-terminal amino acid sequence comprises at least six consecutive amino acid residues chosen from among amino acid residues 247-366 of human fetuin A and amino acid residues 255-382 of human fetuin B.

31. The fusion molecule of 29, wherein the C-terminal amino acid sequence comprises at least six consecutive amino acid residues chosen from among amino acid residues 149-366 of human fetuin A and amino acid residues 157-382 of human fetuin B.

32. The fusion molecule of 1, further comprising a secretion signal sequence.

33. The fusion molecule of 32, wherein the secretion signal sequence is a naturally occurring signal sequence for the therapeutic molecule or prophylactic molecule.

34. The fusion molecule of 32, wherein the secretion signal sequence is a naturally occurring signal sequence for the fusion partner.

35. A polynucleotide encoding the fusion molecule of any of 1-34.

36. A polynucleotide encoding the fusion molecule of 8, wherein the variant comprises a deletion of one or more nucleotides in exon two of human fetuin A.

37. A vector comprising the polynucleotide of 35 or 36 and a regulatory sequence for transcription of the polynucleotide.

38. The vector of 37, wherein the regulatory sequence for transcription is chosen from phage lambda PL promoter, the *E. coli* lac, trp, phoA or tac promoters, the SV40 early or late promoters, adenovirus major late promoter, cytomegalovirus promoter, respiratory syncytial virus promoter, retroviral LTRs, metallothionein promoters, heat shock promoters, albumin promoters, ApoA1 promoter, human globin promoters, viral thymidine kinase promoters, beta-actin promoter and human growth hormone promoters.

39. A host cell comprising one or more of the fusion molecules of any of 1-34, or the polynucleotides of 35 or 36, or the vectors of 37 or 38.

40. A composition comprising the fusion molecule of any of 1-34, the polynucleotide of 35 or 36, the vector of 37 or 38, or the host cell of 39 and a buffer or a pharmaceutically acceptable carrier.

41. A method of making the fusion molecule of 1 comprising:

(a) providing the polynucleotide of 35 or 36; and

(b) expressing the polynucleotide in an expression system to produce the fusion molecule.

42. The method of 41, wherein the expression system is chosen from a cell-free expression system, a prokaryotic expression system, and a eukaryotic expression system.

43. The method of 42, wherein the expression system is a prokaryotic expression system comprising a prokaryote.

44. The method of 43, wherein the prokaryote is a bacterial cell.

45. The method of 42, wherein the expression system is a eukaryotic expression system comprising a eukaryote.

46. The method of 45 wherein the eukaryote is an animal cell.

47. The method of 46, wherein the animal cell is a CHO cell.

48. The method of 46, wherein the animal cell is a COS cell.

49. The method of 46, wherein the animal cell is a 293 cell.

50. The method of 46, wherein the animal cell is a Bowes melanoma cell.

51. The method of 45, wherein the eukaryote is a yeast cell.

52. The method of 51, wherein the yeast cell is a *Saccharomyces* cell or a *Pichia* cell.

53. The method of 45, wherein the the eukaryote is an insect cell.

54. The method of 53, wherein the insect cell is a *Drosophila* S2 cell.

55. The method of 54, wherein the insect cell is a *Spodoptera* Sf9 cell.

56. The method of 45, wherein the eukaryote is a plant cell.

57. A method of producing the polynucleotide of 35 or 36 comprising:

(a) providing the polynucleotide of 35 or 36; and

(b) amplifying the polynucleotide of 35 or 36.

58. The method of 57, wherein the step of amplifying the polynucleotide comprises:

(a) introducing the polynucleotide into a cell; and

(b) culturing the cell under conditions that allow the polynucleotide to be amplified.

59. The method of 57, wherein the step of amplifying the polynucleotide comprises allowing the polynucleotide to undergo a polymerase chain reaction.

60. The fusion molecule of 1, wherein the therapeutic molecule or prophylactic molecule is a cytokine.

[0125] It must be noted that, as used herein, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a subject polypeptide" includes a plurality of such polypeptides and reference to "the agent" includes reference to one or more agents and equivalents thereof known to those skilled in the art, and so forth.

[0126] Further, all numbers expressing quantities of ingredients, reaction conditions, % purity, polypeptide and polynucleotide lengths, and so forth, used in the specification and claims, are modified by the term "about," unless otherwise indicated. Accordingly, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties of the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits, applying ordinary rounding techniques. Nonetheless, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors from the standard deviation of its experimental measurement.

[0127] With respect to ranges of values, the invention encompasses each intervening value between the upper and lower limits of the range to at least a tenth of the lower limit's unit, unless the context clearly indicates otherwise. Further, the invention encompasses any other stated intervening values. Moreover, the invention also encompasses ranges excluding either or both of the upper and lower limits of the range, unless specifically excluded from the stated range.

F. REFERENCES

[0128] The specification is most thoroughly understood in light of the cited references, all of which are hereby incorporated by reference in their entireties. The disclosures of the patents, applications, and other references cited above are also herein incorporated by reference in their entireties. The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which, may need to be independently confirmed.

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V. CLAIM

A fusion molecule comprising a first polypeptide that comprises a first amino acid sequence of a therapeutic molecule or a prophylactic molecule and a second polypeptide that comprises a second amino acid sequence of a fusion partner, wherein the fusion partner is a fetuin polypeptide.

VI. ABSTRACT

Fusion molecules that combine fetuin with a therapeutic polypeptide can have a higher plasma stability than the therapeutic polypeptide in the absence of a fetuin fusion partner. These fusion molecules can combine naturally occurring or genetically engineered fetuin molecules with naturally occurring or genetically engineered therapeutic polypeptides, and can be produced in a variety of prokaryotic and eukaryotic expression systems.

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2521981_2521980       ---------------------------------DPETEEAALVAIDYINQNLPWGYKHTL 27
2521983_4502004       MKSLVLLLCLAQLWGWHSAPHGPGLIYRQPNCDDPETEEAALVAIDYINQNLPWGYKHTL 60
CLN00006206           MKSLVLLLCLAQLWGCHSAPHGPGLIYRQPNCDDPETEEAALVAIDYINQNLPWGYKHTL 60
CLN00107400           MKSLVLLLCLAQLWGCHSAPHGPGLIYRQPNCDDPETEEAALVAIDYINQNLPWGYKHTL 60
NP_001613_NM_001622   MKSLVLLLCLAQLWGCHSAPHGPGLIYRQPNCDDPETEEAALVAIDYINQNLPWGYKHTL 60
                                                        ***************************

2521981_2521980       NQIDEVKVWPQQPSGELFEIEIDTLETTCHVLDPTPVARCSVRQLKEHAVEGDCDFQLLK 87
2521983_4502004       NQIDEVKVWPQQPSGELFEIEIDTLETTCHVLDPTPVARCSVRQLKEHAVEGDCDFQLLK 120
CLN00006206           NQIDEVKVWP-QPSGELFEIEIDTLETTCHVLDPTPVARCSVRQLKEHAVEGDCDFQLLK 119
CLN00107400           NQIDEVKVWPQQPSGELFEIEIDTLETTCHVLDPTPVARCSVRQLKEHAVEGDCDFQLLK 120
NP_001613_NM_001622   NQIDEVKVWPQQPSGELFEIEIDTLETTCHVLDPTPVARCSVRQLKEHAVEGDCDFQLLK 120
                      ********** ************************************************

2521981_2521980       LDGKSSVVYAKCDSSPDSAEDVRKVCQDCPLLAPLNDTRVVHAAKAALAAFNAQNNGSNF 147
2521983_4502004       LDGKFSVVYAKCDSSPDSAEDVRKVCQDCPLLAPLNDTRVVHAAKAALAAFNAQNNGSNF 180
CLN00006206           LDGKFSVVYAKCDSSPDSAEDVRKVCQDCPLLAPLNDTRVVHAAKAALAAFNAQNNGSNF 179
CLN00107400           LDGKFSVVYAKCDSSPDSAEDVRKVCQDCPLLAPLNDTRVVHAAKAALAAFNAQNNGSNF 180
NP_001613_NM_001622   LDGKFSVVYAKCDSSPDSAEDVRKVCQDCPLLAPLNDTRVVHAAKAALAAFNAQNNGSNF 180
                      **** ********************************************************

2521981_2521980       QLEEISRAQLVPLPPSTYVEFTVSGTDCVAKEATEAAKCNLLAEKQYGFCKATLSEKLGG 207
2521983_4502004       QLEEISRAQLVPLPPSTYVEFTVCGTDCVAKEATEAAKCNLLAEKQYGFCKATLSEKLGG 240
CLN00006206           QLEEISRAQLVPLPPSTYVEFTVSGTDCVAKEATEAAKCNLLAEKQYGFCKATLSEKLGG 239
CLN00107400           QLEEISRAQLVPLPPSTYVEFTVSGTDCVAKEATEAAKCNLLAEK--------------- 225
NP_001613_NM_001622   QLEEISRAQLVPLPPSTYVEFTVSGTDCVAKEATEAAKCNLLAEKQYGFCKATLSEKLGG 240
                      ***********************.********************

2521981_2521980       AEVAVTCMVFQTQPVSSQPQPEGANEAVPTPVVDPDAPPSPPLGAPGLPPAGSPPDSHVL 267
2521983_4502004       AEVAVTCTVFQTQPVTSQPQPEGANEAVPTPVVDPDAPPSPPLGAPGLPPAGSPPDSHVL 300
CLN00006206           AEVAVTCTVFQTQPVTSQPQPEGANEAVPTPVVDPDAPPSPPLGAPGLPPAGSPPDSHVL 299
CLN00107400           -------------PVTSQPQPEGANEAVPTPVVDPDAPPSPPLGAPGLPPAGSPPDSHVL 272
NP_001613_NM_001622   AEVAVTCTVFQTQPVTSQPQPEGANEAVPTPVVDPDAPPSPPLGAPGLPPAGSPPDSHVL 300
                         **:*******************************************************
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Figure 1 (continued on next page)

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2521981_2521980       LAAPPGHQLHRAHYDLRHTFMGVVSLGSPSGEVSHPRKTRTVVQPSVGAAAGPVVPPCPG 327
2521983_4502004       LAAPPGHQLHRAHYDLRHTFMGVVSLGSPSGEVSHPRKTRTVVQPSVGAAAGPVVPPCPG 360
CLN00006206           LAAPPGHQLHRAHYDLRHTFMGVVSLGSPSGEVSHPRKTRTVVQPSVGAAAGPVVPPCPG 359
CLN00107400           LAAPPGHQLHRAHYDLRHTFMGVVSLGSPSGEVSHPRKTRTVVQPSVGAAAGPVVPPCPG 332
NP_001613_NM_001622   LAAPPGHQLHRAHYDLRHTFMGVVSLGSPSGEVSHPRKTRTVVQPSVGAAAGPVVPPCPG 360
                      ************************************************************

2521981_2521980       RIRHFKV 334
2521983_4502004       RIRHFKV 367
CLN00006206           RIRHFKV 366
CLN00107400           RIRHFKV 339
NP_001613_NM_001622   RIRHFKV 367
                      *******
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Figure 1 (continued from previous page)

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NP_055190_NM_014375  MGLLLPLALCILVLCCGKLSPPQLALNPSALLSRGCNDSDVLAVAGFALRDINKDRKDGY 60
4996126_4996125      MGLLLPLALCILVLCCGAMSPPQLALNPSALLSRGCNDSDVLAVAGFALRDINKDRKDGY 60
                     ********** :****************************************

NP_055190_NM_014375  VLRLNRVNDAQEYRRGGLGSLFYLTLDVLETDCHVLRKKAWQDCGMRIFFESVYGQCKAI 120
4996126_4996125      VLRLNRVNDAQEYRRGGLGSLFYLTLDVLETDCHVLRKKAWQDCGMRIFFESVYGQCKAI 120
                     ************************************************************

NP_055190_NM_014375  FYMNNPSRVLYLAAYNCTLRPVSKKKIYMTCPDCPSSIPTDSSNHQVLEAATESLAKYNN 180
4996126_4996125      FYMNNPSRVLYLAAYNCTLRPVSKKKIYMTCPDCPSSIPTDSSNHQVLEAATESLAKYNN 180
                     ************************************************************

NP_055190_NM_014375  ENTSKQYSLFKVTRASSQWVVGPSYFVEYLIKESPCTKSQASSCSLQSSDSVPVGLCKGS 240
4996126_4996125      ENTSKQYSLFKVTRASSQWVVGPSYFVEYLIKESPCTKSQASSCSLQSSDSVPVGLCKGS 240
                     ************************************************************

NP_055190_NM_014375  LTRTHWEKFVSVTCDFFESQAPATGSENSAVNQKPTNLPKVEESQQKNTPPTDSPSKAGP 300
4996126_4996125      LTRTHWEKFVSVTCDFFESQAPATGSENSAVNQKPTNLPKVEESQQKNTPPTDSPSKAGP 300
                     ************************************************************

NP_055190_NM_014375  RGSVQYLPDLDDKNSQEKGPQEAFPVHLDLTTNPQGETLDISFLFLEPMEEKLVVLPFPK 360
4996126_4996125      RGSVQYLPDLDDKNSQEKGPQEAFPVHLDLTTNPQGETLDISFLFLEPMEEKLVVLPFPK 360
                     ************************************************************

NP_055190_NM_014375  EKARTAECPGPAQNASPLVLPP 382
4996126_4996125      EKARTAECPGPAQNASPLVLPP 382
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Figure 2